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INHIBITION OF THE BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID AND PROTEIN IN HeLa S3 CELLS BY CUCURBITACINS, GLUCOCORTICOID-LIKE CYTOTOXIC TRITERPENES

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Abstract—Cucurbitacins were found to inhibit the incorporation of radioactive precursors into DNA, RNA and protein in intact and permeabilized HeLa S3 cells. The observed inhibition was rapid and irreversible although the maximal effect (almost complete inhibition) required several hours of cell exposition to the agent. The magnitude of the inhibition was, with some exceptions, nearly the same for all three precursors within the entire range of cucurbitacin concentrations examined. The ID_{50} values (concentrations required to produce half-maximal inhibition of the macromolecule biosynthesis) determined for several cucurbitacins were very close to their respective ED_{50} values (those for half-maximal inhibition of cell proliferation). Parallel with the inhibition of [^{3}H]-labelled precursor incorporations into nucleic acids, cucurbitacin diminished the [^{3}H]thymidine and [^{3}H]uridine nucleotides' pool sizes of HeLa S3 cells. No effect of cucurbitacin on the [^{3}H]leucine pool was observed. The studies presented prove that cucurbitacins inhibit the biosynthesis of DNA, RNA and protein in HeLa S3 cells, and that these inhibitory effects are closely related to the inhibition of HeLa S3 cell proliferation by cucurbitacins. The mechanism of the inhibition is unknown but the obtained results suggest that cucurbitacins act upon an unidentified target, which results in the inhibition of macromolecule biosynthesis. It was also found that these inhibitory effects of cucurbitacins are neither mediated by glucocorticoid receptors nor require replication, transcription or translation.

The potent cytotoxic activity of cucurbitacins, tetracyclic triterpenoids of plant origin, against KB [1, 2] and HeLa [2] cells was documented years ago. Their mode of cytotoxic action, however, remains unknown. In fact, we are aware of only two preliminary studies dealing with this question: one concerning the selective inhibition of the incorporation of thymidine into lymphocytes [3] and the other on the inhibition of anaerobic glycolysis and respiration of Ehrlich ascites tumour cells [4, 5]. Recently we found that cucurbitacins inhibit [^{3}H]cortisol binding to glucocorticoid receptors in cytosol and in intact HeLa cells. Besides, on the basis of correlation studies, we suggested that this effect was probably associated with the cytotoxic action of cucurbitacins [6]. Antiproliferative action of glucocorticoids was found to be accompanied by an inhibition of DNA biosynthesis and, in some cases, of RNA and/or protein biosynthesis [7–10]. Although cucurbitacins were postulated to inhibit selectively DNA synthesis in lymphocytes [3], it seemed important to establish their effect on macromolecule biosynthesis as a necessary preliminary step in the elucidation of the mode of action of cucurbitacins.

We studied the influence of cucurbitacin I and other cucurbitacins (Fig. 1) on the biosynthesis of DNA, RNA and protein in HeLa S3 cells, and explored the role of the observed phenomena in the mechanism of cytotoxic activity of these agents.

MATERIALS AND METHODS

Materials. HeLa S3 cells were purchased from Gibco Bio-Cult (U.K.); fetal calf serum (virus and mycoplasma screened) and Eagle's minimal essential medium were from Flow Laboratories; trypsin and bovine serum albumin were from BDH Chemicals Ltd. (Poole, Dorset, U.K.); penicillin G and streptomycin sulfate were from Polfa (Poland); HEPES was from Serva (Heidelberg, F.R.G.); other biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Cucurbitacins were isolated previously from *Bryonia alba* L. [11]; their purity was checked by TLC* [12], and chromatographically pure compounds were used. Cucurbitacins and steroids were dissolved in ethanol and 2 μl of these solutions was added per 1 ml of medium or assay mixture. The same amount of ethanol was added to control samples.

L-[4,5-^{3}H]Leucine (53–60 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, Bucks., U.K.); [methyl-^{3}H]thymidine (22–25 Ci/

* Abbreviations: TCA, trichloroacetic acid; PCA, perchloric acid; TLC, thin-layer chromatography; S.E.M., standard error of the mean; ID_{50}, concentration of compound required to inhibit by 50% the incorporation of ^{3}H-labelled precursor to macromolecule; ED_{50}, concentration of compound required to inhibit by 50% the growth of HeLa S3 cells.

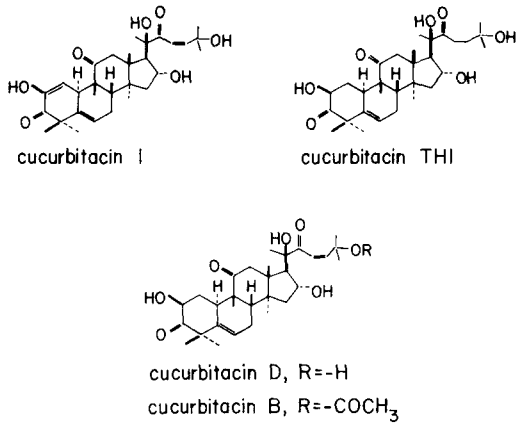

Fig. 1. Structure of cucurbitacins.

mmole), [5-^{3}H]uridine (29 Ci/mmole), [methyl-^{3}H] thymidine-5′-triphosphate (23–27 Ci/mmole) and [5-^{3}H]uridine-5′-triphosphate (26 Ci/mmole) were purchased from UVVVR (Czechoslovakia).

Cell culture. HeLa S3 cells were grown as monolayer cultures in Eagle's minimal essential medium with Earle's salts in the humidified atmosphere of 95% air and 5% CO_2 at 37°. The medium was supplemented with 5% fetal calf serum, 100,000 units/l. penicillin and 100 mg/l. streptomycin sulphate. The cells were passaged as previously described [6].

Measurement of the rate of radioactive precursor incorporation. HeLa S3 cells were plated 16–19 hr prior to use to yield *ca.* 10^5 cells/4 cm^2 Leighton tube at the time of experiments. Sterile solutions of [^{3}H] thymidine, [^{3}H]uridine or [^{3}H]leucine were added at 37° to give final concentrations of 0.5, 0.5 and 3.0 μCi/ml, respectively, and the cells were incubated at 37° for 60 min, unless otherwise indicated. The pulse was terminated by chilling the Leighton tubes in an ice-water bath. The radioactive medium was respirated and the cells were washed 3 times with 2 ml ice-cold PBS (0.14 M NaCl/2.7 mM KCl/0.5 mM $MgCl_2$/0.6 mM $CaCl_2$/1.47 mM KH_2PO_4/7.1 mM Na_2HPO_4/5.5 mM glucose, pH 7.4). The washed cells were removed from the tube surface by treatment with 1 ml 0.1% trypsin–0.2% versene solution for 5–10 min at 37°. Ice-cold 10% w/v TCA (3 ml) was added to the precooled Leighton tube, the tubes were vortexed, and left at 0–4° for 30–60 min. The TCA precipitates were collected on glass-fibre filters (GF/C, Whatman), washed 3 times with 10 ml ice-cold 5% TCA, dried, solubilized in 0.5 ml Nuclear Chicago solubilizer diluted with 3 parts of Tpp4 scintillant, and counted in Tpp4 scintillant containing 1 ml acetic acid per l. [13]. The non-specific binding of radioactive precursors to cellular components was determined after incubation of the cells with labelled precursor at 0–4° for the same time as indicated for the 37° incubations, and the obtained value (2–6% of total radioactivity for control samples) was subtracted from the total radioactivity of the samples. The number of cells and/or the amount of protein were determined for each sample, and the results were normalized by the cell number or the protein amount.

The rates of ^{3}H-labelled precursor incorporation were constant, at least for 90 min of incubation, except for 3–5 min delay for [^{3}H]thymidine and [^{3}H] uridine incorporation, and were proportional to the cell number in the range of cell density 1.0×10^4–6.0×10^4/cm^2 (data not shown). The incorporation rates for [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine were $(7.5 \pm 0.1) \times 10^4$, $(8.1 \pm 0.8) \times 10^4$ and $(1.5 \pm 0.7) \times 10^4$ dpm/10^5 cells per 1 hr ($n = 6$), respectively.

Estimation of the size of the ^{3}H-labelled precursor pool. The total, PCA-soluble and PCA-insoluble radioactivities were determined according to Hauschka [14]. The cells incubated with the ^{3}H-labelled precursor, as described above, were washed 3 times with 1 ml ice-cold PBS for 1 min (0–4°). The PBS-washed cells were hydrolysed in 1 ml 0.4 M PCA for 150 min at 90°, and 0.5 ml ice-cold 0.72 N KOH–0.6 M $KHCO_3$ was added to the precooled tubes. The tubes were vortexed, left for 5 min, and centrifuged at 3000 *g* for 10 min at 2°. The radioactivities of 1 ml aliquot of supernatants were measured in xylene–Triton X-100 scintillant (total radioactivity). For the determination of the PCA-insoluble radioactivity, 10 ml 0.4 M PCA (0–4°) was added to the PBS-washed cells, and the tubes were kept for 30 min at 0–4°. The precipitates were washed 5 times with 2 ml ice-cold 0.4 M PCA, hydrolysed, neutralized and counted as described above. The PCA-soluble radioactivity (^{3}H-labelled precursor pool) was calculated as the difference between the total and PCA-insoluble radioactivity, or estimated directly by ^{3}H-labelled precursor extraction from the PBS-washed cells with 1.5 ml 0.4 M PCA for 30 min at 0–4°. Aliquots (1 ml) of neutralized extract were used for radioactivity measurement as described above. The difference between the sizes of the radioactive precursor pool, estimated directly or calculated, did not exceed 10% of the former value. The size of the ^{3}H-labelled precursor pool of untreated HeLa S3 cells amounted to $(5.1 \pm 0.3) \times 10^5$, $(2.3 \pm 0.3) \times 10^6$ and $(2.92 \pm 0.05) \times 10^5$ dpm/10^6 cells for [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine, respectively.

Incorporation of [^{3}H]thymidine and [^{3}H]uridine into macromolecules of HeLa S3 cells preloaded with labelled precursor. The experimental system used by Plagemann [15] and Woynarowski and Konopa [16] was adapted to monolayer cell culture. Monolayer cultures (130,000 cells per 4 cm^2 Leighton tube, 24 hr after plating) were cooled in an ice-water bath, and the medium was replaced by 1 ml ice-cold 20 mM HEPES-buffered medium (pH 7.4) containing 4 μCi of ^{3}H-labelled precursor and 0.4 μg of non-labelled precursor. Then the tubes were preincubated for 60 min at 3°. The radioactive medium was aspirated and the cells were washed twice with 1 ml ice-cold HEPES-buffered medium. Finally, 1 ml of HEPES-buffered medium and 2 μl of an ethanolic solution of cucurbitacin I or ethanol were added to each tube. The tubes were kept for 10 min at 3°, then 2 min in a water bath at 37°, and incubated at 37° in a CO_2 incubator. After 0, 10 and 20 min of incubation, the total and PCA-insoluble radioactivities were determined as described above.

The ^{3}H-labelled precursor incorporation into untreated HeLa S3 cells was linear for [^{3}H]thymidine

(12,000 dpm/10^5 cells per 10 min) and almost linear for [^{3}H]uridine (27,000 dpm/10^5 cells per 10 min) for 20 min incubation.

Analysis of the [^{3}H]thymidine nucleotide pool of HeLa S3 cells. Except for incubation with cucurbitacin I, all operations were carried at 0–4°. HeLa S3 cells incubated with 1 μM cucurbitacin I or ethanol (control samples) were scraped with a rubber policeman and suspended (10^6 cells/ml) in 20 mM HEPES-buffered medium containing 8 μCi/ml [^{3}H]thymidine and 0.44 μg/ml thymidine. The 5 ml aliquots of cell suspension were placed in siliconized tubes and incubated at 0–4° for 1 hr. After incubation the medium was removed by centrifugation, the cells were extensively washed with cold medium and extracted with 0.2 ml 0.5 M PCA per 5×10^6 cells [15]. The PCA extracts were chromatographed on a polyethylenimine cellulose thin-layer chromatographic plate, 20 × 20 cm (Merck, F.R.G.), pre-developed with water. The chromatogram was developed with 0.2 M LiCl (5.5 cm from the start), then with 0.5 M LiCl (next 5.0 cm), and finally with 0.8 M LiCl (last 9.0 cm). The following R_f values were calculated: 0.15–0.16 for dTTP, 0.25 for dTDP, 0.55 for dTMP, and 0.86 for thymidine. The cellulose zones were removed from the plate, mixed with 1 ml water in scintillation vials, and the radioactivity was determined in 7 ml xylene–Triton X-100 scintillant. The recovery of radioactivity after TLC amounted to 90–95%.

DNA and RNA synthesis in permeable HeLa S3 cells. HeLa S3 cells were plated at 125,000 cells/cm^2 in 4 cm^2 Leighton tubes 16–20 hr prior to experiments. To make the cells permeable, medium was removed and the cells kept in an ice-cold bath, were washed twice with 1 ml ice-cold PBSL (80 mM KCl/4 mM $MgCl_2$/1 mM $CaCl_2$/50 mM sucrose/7.5 mM KH_2PO_4/35 mM HEPES–KOH, pH 7.4 at 0–4°). The cells were thoroughly washed with 0.3 ml cold lysolecithin (L-α-lysophosphatidylcholine, type I, from egg yolk) solution (0.1 mg/ml PBSL), and the tubes were kept for 2 min in an ice-water bath [17]. The permeability of the cells was assessed in separate samples by the uptake of trypan blue (0.1%) for 2 min. The cells were considered permeable when not less than 90% of the cells were stained. Preincubation of the cells with 1 μM cucurbitacin I for 2 hr did not affect the efficiency of permeabilization. A 0.3 ml aliquot of the DNA or RNA synthesis assay mixture (strictly as described by Castellot *et al.* [18] except for the specific activity of [^{3}H]UTP, which was 2 Ci/mmole) was added to each tube with permeabilized cells. After 30 min of incubation at 37° the tubes were cooled (0–4%), the cells were scraped with a rubber policeman and suspended in 1 ml water. 5 ml of an ice-cold 1:1 mixture of saturated solutions of Na_2HPO_4 and $Na_4P_2O_7$ supplemented with solid TCA to 10% w/v and thymidine to 1 mM [13] was added, the tubes were vortexed and kept for 15 min at 0–4°. The precipitates were collected on GF/C glass fibre filters (Whatman), washed 4 times with 10 ml 5% TCA, and finally with 3 × 2 ml of $CHCl_3$–CH_3OH (1:1). The precipitates were solubilized with Nuclear Chicago solubilizer and the radioactivity was counted as described above. The blank radioactivity was determined by incubation of separate samples of permeable cells with the assay mixture for 30 min at 0–4°. This value (25–40% of the total radioactivity of the control sample) was subtracted from the total radioactivity of the permeable cells, and the net value was a measure of the ^{3}H radioactivity incorporated into nucleic acids. The mean values of [^{3}H]dTMP and [^{3}H]UMP incorporated into the control cells amounted to 54.9 ± 1.6 and 10.9 ± 1.1 pmole/10^6 cells per hr, as found in 10–12 experiments.

Other methods. The amount of protein was determined according to Hartree [19], with crystalline bovine albumin as a standard. The radioactivity of the samples was measured in Tpp4 scintillant [20] or in xylene–Triton X-100 based scintillant [21] in an Isocap 300 Liquid Scintillation System (Nuclear Chicago, U.S.A.). Corrections for quenching were made by the sample channel ratio method.

Cell number was determined with a model ZB_i Coulter counter.

RESULTS

Inhibition of ^{3}H-labelled precursor incorporation by cucurbitacin I

The rates of incorporation of [^{3}H]thymidine, [^{3}H]-uridine and [^{3}H]leucine into DNA, RNA and protein, respectively, were inhibited during the 2 hr (Fig. 2a) and 24 hr (Fig. 2b) incubations of HeLa S3 cells with cucurbitacin I. At lower concentrations of cucurbitacin I, the dependence of the inhibition upon cucurbitacin concentration was almost the same for all the tritiated precursors used (Figs. 2a and 2b). As shown, after 2 hr incubation with cucurbitacin I the rates of incorporation of the ^{3}H-labelled precursors into the cells decreased abruptly, reaching a plateau at 30–40% of the untreated-cell rate (Fig. 2a). At higher cucurbitacin concentrations, some reversible diminution of the inhibition of the incorporation rates of [^{3}H]leucine (at 0.1–0.5 μM cucurbitacin I) and [^{3}H]uridine (at 0.5–5 μM cucurbitacin I) was observed (Fig. 2a). After 25 hr incubation with cucurbitacin, the rates of incorporation of the labelled precursors into the cells were almost completely suppressed (Fig. 2b). The time- course of inhibition of the ^{3}H-labelled precursor incorporation at 1 μM cucurbitacin I presented in Fig. 2c shows that after the short lag-period the [^{3}H]thymidine and [^{3}H]leucine incorporation rates decreased rapidly in the first 30 min of agent action. The lower inhibitory activity of cucurbitacin I was observed against [^{3}H]uridine incorporation (Fig. 2c). In contrast, at 10 μM of cucurbitacin I, the kinetics and the magnitude of the rate inhibition of [^{3}H]uridine incorporation were similar to those for [^{3}H]thymidine and [^{3}H]leucine (data not shown). The cucurbitacin-resistant incorporation of the radioactive precursors persisted at an almost unaltered level for several hours and slowly decreased during longer incubation of the cells with the agent (data not shown). The inhibition of the incorporation rates of the tritiated precursors into macromolecules by cucurbitacin I remained at the same level at least for 24 hr after the removal of cucurbitacin I (data not shown).

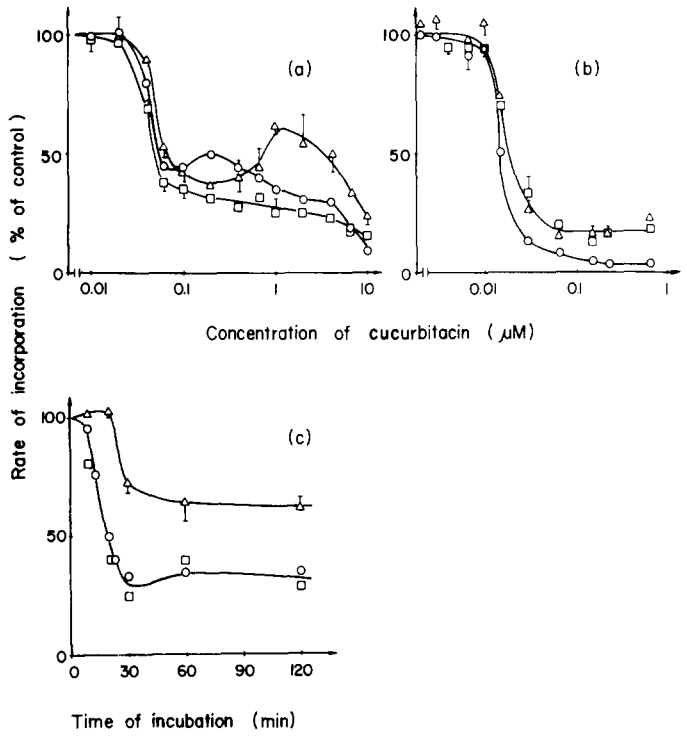

Fig. 2. Influence of cucurbitacin I on the rate of [^{3}H]thymidine (○), [^{3}H]uridine (△) and [^{3}H]leucine (□) incorporation into macromolecules of HeLa S3 cells. (a) Incubation with cucurbitacin for 2 hr. The precursors were incorporated during the last hour of incubation. Cucurbitacin I did not change the protein amount and the cell number compared to the untreated samples. Data are means ± S.E.M. of eight determinations from four experiments. (b) Incubation with cucurbitacin I for 25 hr. The precursors were incorporated during the last hour of incubation. Values were normalized per mg of cellular protein. Data represent means ± S.E.M. for nine determinations from three separate experiments. (c) Time course of the inhibition. ^{3}H-Labelled precursors were added for 30 min ending the incubation with cucurbitacin I at 1 μM. Data represent the average of six determinations from two experiments.

Inhibition of ^{3}H-labelled precursor incorporation by other cucurbitacins

We examined the effect of a series of cucurbitacins (Fig. 1) on the ^{3}H-labelled precursor incorporation into HeLa S3 cells in an attempt to relate the inhibition of the biosynthesis activity to the growth inhibitory action of these compounds. The half-maximal inhibitory concentrations of the cucurbitacins for the inhibition of ^{3}H-labelled precursor incorporation and for growth inhibition (ID_{50} and ED_{50} values, respectively) are compared in Table 1. The ID_{50} values determined after 25 hr incubation and ED_{50} values for each cucurbitacin respectively are almost identical.

Table 1. The ability of cucurbitacins to inhibit the incorporation of ^{3}H-labelled precursors into HeLa S3 cell macromolecules (ID_{50}) and cucurbitacin growth-inhibitory activities against HeLa S3 cells (ED_{50})*

	Incubation time (hr)	Precursor	Cucurbitacin B	I	D	THI
ID_{50} (μM)	2	[^{3}H]Uridine	0.036	0.069	0.39	1.4
	2	[^{3}H]Thymidine	0.020 ± 0.001	0.046 ± 0.003	0.22 ± 0.02	0.78 ± 0.03
	2	[^{3}H]Leucine				
	25	[^{3}H]Thymidine				
	25	[^{3}H]Leucine	0.010 ± 0.001	0.016 ± 0.001	0.040 ± 0.002	0.14 ± 0.01
	25	[^{3}H]Uridine				
ED_{50} (μM)	72	—	0.016	0.021	0.053	0.16

* The ED_{50} values were estimated as previously described [2]. The determination of the amount of cellular protein followed 72 hr incubation of HeLa S3 cells with cucurbitacin. The data are means obtained from two separate experiments. Standard errors of the mean were less than 10% of the estimated value and are omitted from the table.

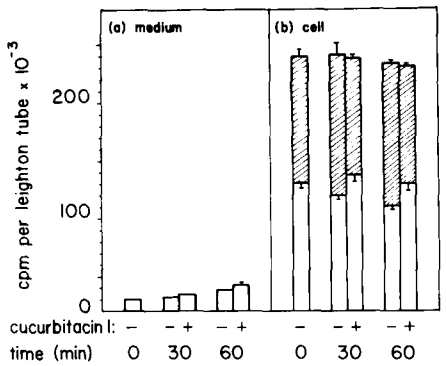

Fig. 3. Changes in the radioactivity pattern of [^{3}H]uridine prelabelled HeLa S3 cells during incubation with cucurbitacin I. 140,000 cells were labelled with [^{3}H]uridine (10 μCi/ml) for 30 min, washed with the medium, and incubated with uridine (25 μg/ml) for 60 min. The medium was replaced with a new medium, cucurbitacin I to 1 μM or ethanol was added, and Leighton tubes were incubated for the indicated times. All operations were carried out at 37°. The tritium released into the medium (a) and the radioactivities of PCA-soluble (open columns) and of PCA-insoluble (black columns) fractions of the cells (b) were measured as described in Materials and Methods in estimation of the size of the ^{3}H-labelled precursor pool. The data represent means ± S.E.M. of three determinations.

The absence of a damaging effect of cucurbitacin I on the cell membrane

The observed potent inhibitory effects of cucurbitacins on the incorporation of ^{3}H-labelled precursor to macromolecules could be an artefact caused by the disruption of the cell membrane. Therefore we ascertained whether cucurbitacin I caused damage to the cell membrane. The trypan blue exclusion test showed that the outer plasma membrane was not permeable to the stain after 2 and 25 hr incubation with 1 μM cucurbitacin I (96 ± 2 and 92 ± 2% cells not stained, respectively), i.e. the cells remained 'alive'.

In order to examine the cell membrane damaging effect of cucurbitacin I, we also measured the cell's ability to retain the acid-soluble pool of [^{3}H]uridine nucleotide, as well as of RNA [22]. The data in Fig. 3 show only a slightly higher efflux of [^{3}H]uridine-derived radioactivity from cucurbitacin-treated cells than from untreated cells (Fig. 3a), and the concurrent changes in the radioactivity of the cellular acid-insoluble and acid-soluble fractions of the cells (Fig. 3b). Under these conditions, the rates of [^{3}H]thymidine and [^{3}H]uridine incorporation were diminished by cucurbitacin I to 30–40% of the value for untreated cells (Fig. 2a).

The effect of cucurbitacin I on the ^{3}H-labelled precursor pool sizes

It has been reported that exogenous thymidine and uridine are not essential factors for HeLa cell growth [23]. The inhibitor-diminished supply of [^{3}H]-uridine or [^{3}H]thymidine into cells, caused, for example, by inhibition of thymidine or uridine kinase salvage pathways, suppresses the incorporation of labelled precursor into nucleic acid without disturbing the biosynthesis of this macromolecule. In order to determine whether the inhibitory effect of cucurbitacin I on the incorporation of labelled precursors into macromolecules is a consequence of cucurbitacin-induced inhibition of respective transport systems, we studied the effect of this agent on ^{3}H-labelled precursor pool sizes. The results in Table 2 show that cucurbitacin I had no influence on the tritiated leucine pool which together with the inhibition of [^{3}H]leucine incorporation indicates strong inhibitory activity of cucurbitacin against protein biosynthesis in HeLa S3 cells. On the contrary, after incubations of 60 and 120 min cucurbitacin I diminished the sizes of the ^{3}H-labelled nucleotide pools in proportion to the degree of inhibition of the radioactivity incorporated into nucleic acids.

Although under certain conditions (30 min incubation with 1 μM cucurbitacin I) the inhibition of the rate of [^{3}H]thymidine incorporation was not accompanied by any decrease in the precursor pool size (Table 2), the incorporation data cannot be interpreted directly as inhibition of biosynthesis of nucleic acids. The effect of cucurbitacin I on [^{3}H]thymidine incorporation may have resulted from the impaired metabolic conversion of thymidine. In an attmept to exclude this possibility, we determined the cucurbitacin influence on [^{3}H]thymidine phosphorylation in HeLa S3 cells. It had been previously demonstrated [14, 24] that the incorporation of thymidine into the intracellular nucleotide pool of HeLa cells proceeded at 0–4°, whereas no incorporation of the precursor into DNA occurred at 4° [16]. In our experiments

Table 2. The size of the ^{3}H-labelled precursor pool of cucurbitacin I-treated HeLa S3 cells*

Incubation time (min)	Precursor	Pool (% of control) Cucurbitacin I (μM) 0.04	1.0	Incorporation (% of control) Cucurbitacin I (μM) 0.04	1.0
30	[^{3}H]Thymidine	—	113 ± 21 (9)	—	31 ± 4 (3)
60	[^{3}H]Thymidine	—	60 ± 4 (6)	—	35 ± 1 (3)
120	[^{3}H]Thymidine	89 ± 6 (4)	62 ± 5 (6)	78 ± 4 (4)	36 ± 3 (6)
120	[^{3}H]Uridine	94 ± 1 (4)	84 ± 3 (4)	90 ± 2 (4)	68 ± 3 (4)
120	[^{3}H]Leucine	86 ± 2 (4)	99 ± 2 (4)	75 ± 2 (4)	37 ± 3 (4)

* The cells were incubated with cucurbitacin I at 37° for the times indicated. Tritiated thymidine, uridine or leucine at 1, 1 or 5 μCi/ml, respectively, was added for the last 15 min. PCA-soluble (pool) and RCA-insoluble (incorporation) radioactivities were determined as described in Materials and Methods. The values represent the means ± S.E.M. Of three determinations from two or three experiments. The number of determinations is indicated in parentheses.

Table 3. The effect of cucurbitacin I on the [^{3}H]thymidine nucleotide pool of HeLa S3 cell*

	Size of [^{3}H]thymidine nucleotide pool (pmole/10^6 cells)	Distribution of [^{3}H]thymidine (%)			
		dT	dTMP	dTDP	dTTP
Untreated cells	7.84 ± 0.01 (2)	5.3	56.5	8.7	29.6
Cells incubated with cucurbitacin I (1 μM)					
At 0–4°	10.80 ± 0.01 (2)	2.6	57.5	8.1	31.8
At 37°	3.76 ± 0.09 (2)	6.2	38.9	22.4	32.6

* The cells grown in 48 cm^2 Leighton tubes were incubated with 1 μM cucurbitacin I for 2 hr at 0–4° or 37°, then with 8 μCi/ml [^{3}H]thymidine (4.1 Ci/mmole) for 1 hr at 0–4°.

Subsequent analysis of the distribution of the [^{3}H]thymidine nucleotide was carried out as described in the Materials and Methods.

HeLa S3 cells were first incubated with cucurbitacin I at 0–4° or 37° and then with [^{3}H]thymidine at 0–4°. The PCA-soluble radioactivity was extracted from the cells and chromatographed on PEI-cellulose. This mode of operation enabled the determination of the activity of the exogeneous precursor salvage pathway. The HeLa S3 cells when preincubated with cucurbitacin I at 37° diminished the [^{3}H]thymidine nucleotide pool size (Table 3). Analysis of the [^{3}H]-thymidine nucleotide distribution pattern of cucurbitacin-treated cells showed an increase amount of [^{3}H]-dTDP and a reduced amount of [^{3}H]dTMP compared with the control cells.

No decrease in the [^{3}H]thymidine nucleotide pool size was observed when HeLa S3 cells were preincubated with cucurbitacin I at 0–4° (Table 3).

Cucurbitacin inhibition of DNA and RNA biosyntheses

The data presented in Table 2 illustrating the cucurbitacin I inhibition of the pool sizes of ^{3}H-labelled nucleotides and of the ^{3}H-labelled nucleotide incorporation into HeLa S3 cells do not prove equivocally that cucurbitacin inhibits the biosynthesis of nucleic acids. In an attempt to establish the ability of cucurbitacin to inhibit nucleic acid biosynthesis, we applied an experimental system which enabled direct analysis of the immediate effects of a compound on nucleic acid biosynthesis avoiding a simultaneous conversion of exogenously supplied ^{3}H-labelled nucleosides into ^{3}H-labelled nucleotides [15, 16]. The HeLa S3 cells were preincubated with [^{3}H]thymidine or [^{3}H]uridine at 0–4° to build up the intracellular [^{3}H]thymidine and [^{3}H]-uridine nucleotide pools (Table 2 and ref. [15]). Then the cells were washed and incubated at 37° in order to determine the ability of the cells to synthesize radioactive nucleic acids. Cucurbitacin I added to the preloaded cells caused, after a 10 min lag-period, an equally effective inhibition of both DNA and RNA biosyntheses (Fig. 4).

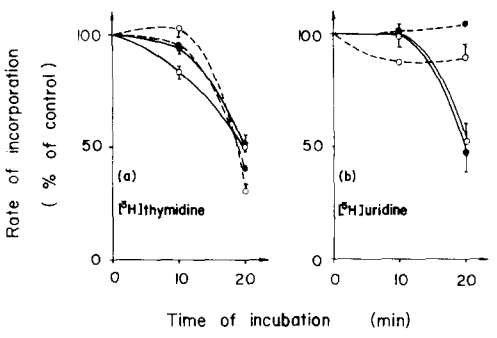

Fig. 4. Effect of cucurbitacin I on the incorporation of interacellular [^{3}H]thymidine (a) and [^{3}H]uridine (b) into nucleic acids of HeLa S3 cells. The cells were preloaded with ^{3}H-labelled precursor at 3° and incubated at 37° with cucurbitacin I at 1 (●) or 10 μM (○) in fresh medium without labelled precursor (solid lines). The broken lines represent inhibition of the incorporation of tritium into HeLa S3 cells when cucurbitacin I and radioactive precursor were added simultaneously just before the incubation at 37° (standard system). The data represent means ± S.E.M. of six determinations from two experiments.

To exclude the possibility that suppression of triphosphate nucleotide synthesis by cucurbitacin promotes the inhibition of nucleic acid biosynthesis, the effect of cucurbitacin I on the biosynthesis of DNA and RNA was investigated using permeabilized cells, the system described by Miller *et al.* [17, 25]. Untreated and cucurbitacin I-treated cells were first permeabilized using lysolecithin (100 μg/ml, 0–4°, 2 min), then incubated with the DNA or RNA synthesis mixture at 37° as described in Materials and Methods. The 2 hr preincubation of HeLa S3 cells with cucurbitacin I resulted in the inhibition of the rate of [^{3}H]dTTP or [^{3}H]UTP incorporation into DNA and RNA, respectively, as shown in Fig. 5, thereby confirming the ability of cucurbitacin I to inhibit nucleic acid biosynthesis in HeLa S3 cells.

Cucurbitacin I (0.1–1 μM) added to permeabilized HeLa S3 cells directly with the DNA or RNA synthesis mixture did not inhibit nucleic acid biosynthesis after incubation at 37° for 30 min (Fig. 5) or 1 hr (data not shown).

Influence of transcription and translation inhibitors on the inhibitory activity of cucurbitacin I

We wondered whether any of the cucurbitacin inhibitory activities was a primary event with respect to the remaining ones. Therefore, we analysed the influence of actinomycin D and cycloheximide, inhibitors of transcription and translation respectively, on the action of cucurbitacin I. To characterize the

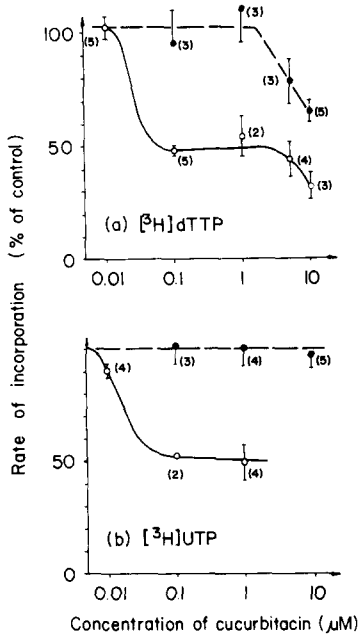

Fig. 5. Influence of cucurbitacin I on [^{3}H]dTTP (a) and [^{3}H]UTP (b) incorporation into lysolecithin permeabilized HeLa S3 cells. The cells were preincubated with cucurbitacin I at 37° for 2 hr before permeabilization (○) or cucurbitacin I was supplied with the assay mixture to permeable cells (●). The data represent means ± S.E.M. from two experiments.

effects of sole inhibitors, the HeLa S3 cells were first incubated with actinomycin D at 1 μg/ml, or cycloheximide at 10 μg/ml, for 20 min and then for 15 min with a tritiated precursor. Under such conditions, actinomycin D blocked selectively and almost completely (95%) [^{3}H]uridine incorporation while cycloheximide caused 60% inhibition of [^{3}H]-leucine incorporation into cells with a small effect on [^{3}H]uridine incorporation. Incubation of the cells with cycloheximide at concentrations higher than 10 μg/ml resulted in an increase of inhibition of labelled uridine incorporation into RNA without any further increase of inhibition of [^{3}H]leucine incorporation into cellular proteins (data not shown).

The experiments with cucurbitacin I proved that its inhibitory effects on [^{3}H]thymidine and [^{3}H]leucine incorporation were not abolished by the actinomycin D-block of transciption (Table 4). Similarly, the 60% inhibition of protein biosynthesis by cycloheximide did not effect the cucurbitacin-caused inhibition of [^{3}H]uridine incorporation into RNA (Table 4).

Inhibition of protein biosynthesis by cucurbitacin I in non-proliferating HeLa S3 cells

In an attempt to evaluate the role of cell proliferation and DNA biosynthesis in the mediation of inhibitory effects of cucurbitacins, we performed experiments on non-proliferating HeLa S3 cells. Cell proliferation and [^{3}H]thymidine incorporation into HeLa S3 cells were almost completely stopped by cell contact inhibition. It was found that the incorporation of [^{3}H]thymidine and [^{3}H]leucine into growth-arrested cells (at density 790,000 ± 2000 cells/cm^2) amounted to 4 and 20% respectively of the values for exponentially growing cells.

Exposition of the cell contact inhibited HeLa S3 cells to cucurbitacin I for 2 hr led to a concentration-dependent decrease of the rate of protein biosynthesis characterized by $ID_{50} = 0.38 \pm 0.02$ (4) μM, while the cucurbitacin-resistant incorporation amounted to 14% of that for untreated cells.

The effect of glucocorticoids on the inhibitory action of cucurbitacins

Our previous results [6] suggested that cucurbitacins affected the cell growth by interaction with glucocorticoid receptors. In an attempt to determine whether the observed inhibitory action of cucurbitacins on the intracellular biosynthesis activity is mediated via a glucocorticoid receptor-dependent mechanism, we examined the influence of glucocorticoids (cortisol and dexamethasone) and antiglucocorticoids (cortexolone and progesterone) on the cucurbitacin-caused inhibition of the rate of ^{3}H-labelled precursor incorporation into HeLa S3 cells. The steroids capable of competing with cucurbitacin for glucocorticoid binding sites could be expected to decrease the effect of cucurbitacins on macromolecule biosynthesis, provided that this binding played a substantial role in the cucurbitacin action. As seen in Table 5, the steroids alone did not significantly

Table 4. The lack of effect of actinomycin D and cycloheximide on the ability of cucurbitacin I to inhibit the ^{3}H-labelled precursor incorporation into HeLa S3 cells*

Inhibitor	Tritiated precursor incorporated	% of control incorporation Cucurbitacin I Alone (1 μM)	With inhibitor
Actinomycin D (1 μg/ml)	[^{3}H]Thymidine	66 ± 1	62 ± 2
	[^{3}H]Leucine	53 ± 4	52 ± 4
Cycloheximide (10 μg/ml)	[^{3}H]Uridine	68 ± 2	65 ± 5

* The block of macromolecule biosynthesis was realized by preincubation of HeLa S3 cells (in 4 cm^2 Leighton tubes) with actinomycin D or cycloheximide for 20 min at 37°. The cells were incubated for an additional 20 min with cucurbitacin I. Tritiated thymidine, uridine or leucine (1, 1, or 5 μCi/ml, respectively) was present during the last 15 min of incubation. All operations were carried out at 37°. The values represented means ± S.E.M. of six determinations from two experiments.

Table 5. The lack of effect of glucocorticoids on cucurbitacin I inhibition of [^{3}H] leucine incorporation into HeLa S3 cells*

Steroid	[^{3}H]Leucine incorporation (% of control) Steroid alone (1 μM)	Cucurbitacin I (0.1 μM) and steroid (1 μM)
None	100 ± 4	19 ± 1
Cortisol	113 ± 5	20 ± 1
Dexamethasone	103 ± 4	16 ± 2
Cortexolone	88 ± 1	15 ± 1
Progesterone	109 ± 1	15 ± 2

* Ethanolic solutions of steroids were added to HeLa S3 cell cultures grown in 4 cm^2 Leighton tubes and kept at 37°. After 5 min cucurbitacin I was added. The cells were incubated for 2 hr. [^{3}H]Leucine (3 μCi/ml) was present during the last 30 min of incubation. All operations were carried at 37°. The data represent means ± S.E.M. of six determinations from two experiments.

influence the rate of [^{3}H]leucine incorporation, and when given to cells together with cucurbitacin I did not prevent cucurbitacin inhibition of the rate of [^{3}H]-leucine incorporation into protein. Similar results were obtained for [^{3}H]thymidine incorporation into HeLa S3 cells (data not shown).

DISCUSSION

Cucurbitacin I, one of the most cytotoxic cucurbitacins, was shown to inhibit the rate of incorporation of radioactive precursors into DNA, RNA and protein (Figs. 2a and 2b). The observed inhibition was rapid (Fig. 2c), irreversible, and almost complete after several hours of cell treatment (see Results). Both the inhibition of [^{3}H]leucine incorporation into protein and the lack of effect on the [^{3}H]leucine pool (Table 1) imply the suppression of protein biosynthesis by cucurbitacin I. Likewise, the data obtained from the studies on cells preloaded with radioactive precursor (Fig. 4) and on permeabilized cells (Fig. 5) indicate that cucurbitacin I also inhibits the biosynthesis of nucleic acids. The inhibitory effect of cucurbitacin I on DNA synthesis in these two systems (Figs. 4 and 5) was very closely related to that observed in the standard system (Fig. 2a). On the other hand, the inhibition of RNA biosynthesis by 0.5–5 μM cucurbitacin I was more efficient in the preloaded and permeabilized cells (Figs. 4 and 5) than the inhibition of [^{3}H]uridine incorporation in the standard system (Fig. 2a). It is likely that the effect of cucurbitacin I in the latter case was partly concealed by some enhancement of the uptake of [^{3}H]uridine into a pool of uridine nucleotides.

Similar parts of each DNA-, RNA- and protein synthesis were found to be resistant to cucurbitacin inhibition (Figs. 2a, 4 and 5). This fact, together with the previously discussed results, strongly suggests that the inhibition of each macromolecules biosynthesis by cucurbitacin arises from a common source.

The ID_{50} values determined after 25 hr incubation, and ED_{50} values for several cucurbitacins were very close to each other, respectively (Table 1). Thus a strong relationship is likely to exist between the inhibition of intracellular metabolic activity and growth-inhibitory action of cucurbitacins.

The possibility that these inhibitory effects were caused by cell lysis was excluded on the basis of the trypan blue test (see Results) and on the ability of the cucurbitacin-treated cells to retain the intracellular [^{3}H]uridine pool (Fig. 3).

The inhibition of [^{3}H]thymidine incorporation by cucurbitacin I was accompanied by the diminution of the [^{3}H]thymidine nucleotide pool size (Table 2). Under specific conditions (30 min incubation at 37°), however, [^{3}H]thymidine incorporation was inhibited by cucurbitacin I without any effect on the [^{3}H]-thymidine nucleotide pool size (Table 2). No inhibitory effect of cucurbitacin I on the [^{3}H]thymidine nucleotide pool of HeLa S3 cells was observed when the cells were incubated with cucurbitacin at 0–4° (Table 3). Moreover, the temperature-dependent decrease of the amounts of each of the [^{3}H]thymidine nucleotides was observed when the [^{3}H]thymidine nucleotide pool was diminished (Table 3). Hence it can be suggested that the diminution of the [^{3}H]-thymidine nucleotide pool size by cucurbitacin may result from feedback inhibition of thymidine phosphorylation by dTTP following the suppression of DNA biosynthesis by cucurbitacin.

Preincubation of intact HeLa S3 cells with cucurbitacin I at 37° followed by cell permeabilization suppressed the incorporation of [^{3}H]dTTP and [^{3}H]UTP into nucleic acids (Fig. 5). However, no effect of cucurbitacin I on [^{3}H]dTTP and [^{3}H]UTP incorporation was observed when the compound was added to permeabilized cells (Fig. 5). These results indicate that cucurbitacin does not directly affect the processes which are inhibited by it. Two possibilities may explain this suggestion: (1) cucurbitacins may undergo metabolic transformation to an active form in intact cells [6] but not in permeabilized cells; and (2) the permeabilized cells are destitute of some cellular activities which are necessary to mediate the inhibitory action of cucurbitacin I.

Whitehouse and Doskotch reported [3] that cucurbitacins selectively inhibited [^{3}H]thymidine incorporation into lymphocytes after brief (30 min) incubation. Our results for HeLa S3 cells do not confirm their findings. The mode of cucurbitacin action on labelled precursor incorporation into macromolecules may differ in HeLa S3 cells and in lymphocytes. The selective inhibition of [^{3}H]thymidine incorpora-

tion observed by Whitehouse and Doskotch could arise from the inhibition of [^{3}H]thymidine uptake without any effect on DNA synthesis, as found for the action of some triterpenoids on phytohaemagglutinin-stimulated lymphocytes [26] and for the glucocorticoid effect on HeLa S3 cells [27].

We demonstrated previously the reversible and concentration-dependent cucurbitacin inhibition of [^{3}H]cortisol binding to glucocorticoid receptors in HeLa cells and in a cell-free system derived from HeLa cells [6]. This effect was found to be correlated with the cucurbitacin growth-inhibitory activity and therefore it was suggested to be a necessary step for growth inhibitory action of these compounds. Recently we established that the ability of cucurbitacins to inhibit the growth of three cultured hepatoma cell lines is related to the sensitivity of these cell lines to the antiproliferating action of dexamethasone (A. Witkowski, A. Venetianer and J. Konopa, unpublished results). These findings indicated that the binding to glucocorticoid receptors mediates the inhibitory effects of cucurbitacins. In the studies presented here we examined the effects of glucocorticoids and antiglucocorticoids (competitors to glucocorticoid receptors) on the cucurbitacin-caused inhibition of the ^{3}H-labelled precursor incorporation into DNA and protein of HeLa S3 cells. The concentration of the steroids used to compete with cucurbitacin I was large enough to ensure complete inhibition of the cucurbitacin binding to the receptors since previously determined relative binding affinity of cucurbitacin I to glucocorticoid receptors amounted to 0.025 of the cortisol affinity [6]. None of the examined steroids altered the cucurbitacin inhibition of the rate of ^{3}H-labelled precursor incorporation into macromolecules (Table 5). Hence these results do not indicate the mediating role of glucocortoid receptors in the inhibition of macromolecule synthesis by cucurbitacins.

Each of the inhibitory effects of cucurbitacins on macromolecule biosynthesis could be sufficient in itself to account for the other ones. The experiments with transcriptional and translational inhibitors (Table 4 and Results) demonstrated that the inhibitory activities of cucurbitacin in HeLa S3 cells do not depend on transcription and translation; the latter, however, being unequivocal since cycloheximide did not block translation completely (see Results), and the remaining protein synthesis might be sufficient to mediate the cucurbitacin effects. On the other hand, the ability of cucurbitacin I to cease protein biosynthesis in cell contact inhibited HeLa S3 cells (see Results) indicated that cell proliferation and replication were not necessary for cucurbitacin inhibition of protein biosynthesis.

On the basis of our results, it seems that the inhibitory effects of cucurbitacin on the biosynthesis of cellular macromolecules, as well as the inhibition of cellular growth, originate from a common, unknown target of the cucurbitacin action. The results obtained rule out the mediatory role of glucocorticoid receptors. However, the correlation between growth-inhibitory activity of cucurbitacins and dexamethasone against hepatoma cells lines characterized by different sensitivity to glucocorticoids (A. Witkowski, A. Venetianer and J. Konopa, unpublished results) strongly suggests a glucocorticoid-like mechanism of cucurbitacin growth-inhibitory action. The apparent contradiction could be explained by the assumption that the cucurbitacin inhibitory action on biosynthesis involves a mechanism resembling the immediate extragenomic effects of glucocorticoids [28–31] which are not necessarily receptor-mediated.

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